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#25

Applicant:	Dieter C. Gruenert et al.	Examiner:	B. Loeb
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Title:	GENE THERAPY BY SMALL FRAGMENT HOMOLOGOUS REPLACEMENT		

CERTIFICATE OF MAILING OR TRANSMISSION UNDER 37 CFR 1.8

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By:
Name: Karen S. Canady

DECLARATION OF DIETER C. GRUENERT UNDER 37 C.F.R. §1.132

I, DIETER C. GRUENERT, declare as follows:

Not Considered 8/13/03

1. I am a named inventor on the patent application identified above, and am authorized by the Assignee to make this declaration.

2. I am a Professor of Medicine, Director of the Human Molecular Genetics Unit, and a member of the Vermont Cancer Center, at the University of Vermont. I earned a Ph.D. in radiation biophysics in 1982 from the University of California, Berkeley, after which I was a postdoctoral fellow with Dr. P.A. Cerutti, Department of Carcinogenesis, Institut Suisse de Recherches Experimentales sur le Cancer, Epalinges/Lausanne, Suisse. In 1984, I joined the private sector as a molecular biologist with the therapeutic division of Hana Biologics, Inc., in Berkeley, California. I then returned to academia in 1986 as an assistant research biochemist in the Cardiovascular Research Institute at the University of California, San Francisco, where I became an associate professor in 1993. I served as co-director of the UCSF Gene Therapy Core Center for seven years before joining the faculty of the UVM College of Medicine in 2000. I have been a consultant and advisor to several biotechnology companies, as well as an ad hoc reviewer for the NIH. My extensive research experience includes the elucidation of the biochemical and genetic mechanisms underlying abnormalities in human genetic disorders as well as human airway epithelial cell and cancer biology. I initiated studies and development of the novel

approach to gene targeting described in the above-identified patent application. In addition to developing strategies for gene therapy of cystic fibrosis and sickle cell anemia as well as other monogenic and multigenic diseases, this work has also led me to investigations into the development of transgenic large animal models of disease. This work has been coupled with studies investigating non-viral delivery systems ranging from liposomes, polyamidoamines, polyethyleneimines, polypeptides, microinjection, and electroporation.

3. I have reviewed the above-identified patent application and the Office Action dated March 26, 2003, issued in connection with the above-identified patent application.

4. The methods described and claimed in the above-identified patent application are in fact successful in replacing a target fragment of a gene in a cell. More specifically, work in my lab and work by others (summarized below) has succeeded in delivering to a cell, both *in vivo* and *ex vivo*, an exogenous replacement DNA fragment. The exogenous replacement DNA fragments used in these studies consisted of one or more replacement exons flanked by noncoding sequence at both the 3' and 5' ends, with the noncoding sequence flanking the replacement exons being homologous to noncoding sequence flanking the target fragment of the gene in the cell. The replacement DNA fragments have been successfully delivered to cells using both lipid-based (lipofectamine, lipofectin®) and mechanical delivery systems (microinjection, electroporation). Successful replacement was confirmed by both structural and functional analyses. This fragment replacement strategy is referred to herein as small fragment homologous replacement, or "SFHR".

5. In addition to the published work described in the Amendment submitted in connection with the above-identified patent application on December 3, 2001 (see page 18 and accompanying exhibits), more recent work has confirmed that SFHR results in (1) replacement of the target fragment with the replacement fragment in both *in vivo* and *ex vivo* model systems; (2) complete replacement at both alleles (Exhibit A submitted herewith); (3) subsequent expression of the replacement fragment in human hematopoetic stem/progenitor cells (HSPC) following SFHR-mediated modification of β-globin sequences (Exhibit B); (4) stable inheritance of the replacement fragment at the chromosomal level (Exhibit B); and (5) functional correction of ion transport properties

in the nasal mucosa in transfected mice in an *in vivo* model of cystic fibrosis (Paragraph 6, below). In addition, the HSPC carrying β-globin sequences modified by SFHR *ex vivo* were capable of engrafting immune deficient mice. The SFHR-mediated modification of β-globin sequences in the *ex vivo* system is described in two manuscripts recently submitted for publication and appended hereto as Exhibits A (Prokopishyn et al.) and B (Goncz et al.). The SFHR-mediated modification of ion transport in nasal mucosa in a mouse model of cystic fibrosis is described in paragraph 6 below.

6. In recent studies that I carried out in collaboration with Drs. William Colledge, Martin Evans, and Alan Cuthbert at Cambridge University, UK, short circuit current measurements were made of nasal mucosa from ΔF508/ΔF508 mice (an animal model for cystic fibrosis) transfected with 786-bp fragments of mouse genomic (wild type) cystic fibrosis transmembrane conductance regulator (CFTR). Animals were transfected with lipofectamine DNA complexes. Prior to complexing with the lipid, the DNA fragments were denatured by boiling at 95°C for 10 min and immediately cooling the DNA in ice water. Lipid-DNA complexes (100 μl) were added to the nose in 2-5 μl aliquots. Nasal mucosa were harvested 2 days after transfection (Experiment 1), 2 days after 4 consecutive applications (Experiments 2 and 3), and (*) 10 days after 4 applications (Experiment 4). Experiment 1: a) I_{sc} from 3 control animals (5 tissue samples) transfected with lipid alone. b) I_{sc} from 5 animals (7 tissue samples) transfected with lipid-wt fragment complexes. Experiments 2 - 4: a) I_{sc} from 5 control animals (5 tissue samples) transfected with lipid alone. b) I_{sc} from 5 animals (5 tissue samples) transfected with lipid-wt fragment complexes.

Analysis of the ion transport properties of the transfected ΔF508/ΔF508 mice indicated a mean increase in Cl^- ion transport greater than that observed with the lipid controls (Table 1). The analysis of the ion transport properties of transfected ΔF508 mice indicated a mean increase in Cl^- ion transport greater than that observed with the lipid controls in 2 different experiments. These studies indicate that delivery of a wild-type mCFTR fragment into the nasal mucosa of ΔF508 CF mice will change the cAMP-dependent ion transport properties of the mice, in that they now secrete Cl^- in response to cAMP stimulation. Success with 2 different experiments is remarkable, given the limited availability of the ΔF508 animals and the variability of the parameters of the system (e.g., animal age, size, sex, feeding time before the experiment). In the 2 experiments where a significant increase in

the cAMP stimulated I_{sc} was observed, the level of cAMP-dependent Cl transport was within a range comparable to that observed in normal animals.

Table 1: SHORT CIRCUIT CURRENT AFTER SFHR

Experiment	I_{sc} (- Frag) μA/cm²	I_{sc} (+wt Frag) μA/cm²
1	8±4.8 (n=7)[a]	23.1 ±5.0 (n=5)[b]
2	2.4±0.5 (n=5)[a]	4.5±2.3 (n=5)[b]
3		32.5±10.0 (n=5)[b]
4*		2.6±1.4 (n=5)[b]

7. Contrary to the statements at pages 6-9 of the Office Action dated March 26, 2003, it has been established that the SFHR method, practiced in accordance with the teachings in the specification of the application as filed, is capable of achieving replacement of a target fragment of a gene, and at levels sufficient for both animal models for study of disease and for therapeutic benefit. The examples of successful SFHR mentioned in the preceding paragraphs used two different gene fragment delivery strategies: microinjection and lipid-based delivery. Both of these strategies are described in the specification (e.g., microinjection at page 23, lines 25-30; and lipid-based delivery at page 42, line 11, at page 73, line 12, and at page 75, line 32, to page 76, line 14). In addition, the variety of suitable delivery approaches is discussed in the specification at page 24, lines 25-29, and at page 40, as well as throughout the Examples portion. Accordingly, the person skilled in the art as of the filing date of this application could have practiced the claimed invention by following the teachings of the specification.

8. I further declare that all statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 5.25.03

Dieter C. Ciminert, Ph.D.

EXHIBIT A

TARGETED GENOME EDITING OF THE β-GLOBIN GENE IN ENGRAFTING HUMAN BLOOD STEM CELLS

Nicole L. Prokopishyn[1,2], Judith Yannariello-Brown[1], Kaarin K. Goncz[3], Barbara L. Chow[1], Dieter C. Gruenert[3], and Brian R. Davis[1,2,*]

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ABSTRACT

Ex vivo correction of disease-causing genetic mutations in stem cells, followed by autologous transplantation, represents a promising therapeutic approach for genetic diseases, including hemoglobinopathies such as sickle cell anemia and thalassemia. Crucial for the success of this approach will be the ability to introduce targeted, sequence-specific heritable changes correcting the genetic mutations in organ-specific stem cells, while retaining the stem cell activity of the modified cells. In this report, we provide evidence that nuclear-specific delivery of small DNA fragments (SDF) effected conversion of the normal β-globin gene to the sickle cell genotype (an A to T substitution within codon 6) in human primitive hematopoietic stem/progenitor cells. The successful engraftment of these small fragment homologous replacement (SFHR)-modified cells in immune deficient mice demonstrated both efficient and stable conversion of wild-type β-globin in human blood stem/progenitor cells, with retention of stem cell activity. These findings are very promising with respect to the application of targeted gene correction in the treatment of genetic disease.

Keywords: gene therapy, sickle cell anemia, gene repair, hematopoietic stem cell

INTRODUCTION

Sickle cell anemia is a hereditary hemoglobin disorder caused by a point mutation (A to T transversion) in the sixth codon of the β-globin gene [1]. A major consideration in the treatment of any hemoglobinopathy is the maintenance of the tightly regulated, physiological, and erythroid-specific expression of the normal globin gene cluster [2]. Three possible approaches can be envisioned for gene therapy treatment of hemoglobinopathies such as sickle cell anemia – delivery of a replacement copy of the relevant globin gene (e.g. β-globin or γ-globin) [3], introduction of a specially designed anti-sickling globin[4], or actual repair of the globin gene. The complex and coordinate regulation of globin gene expression *in vivo* raises significant difficulties in treating sickle cell anemia by addition of a normal or specially designed globin gene using retroviral, lentiviral, or adeno-associated viral vectors[5]. Repairing the mutation itself within the β-globin gene would maintain the corrected genetic material within its normal chromatin environment and in principle, ensure appropriate genetic regulation and expression in the progeny erythroid cells.

Small Fragment Homologous Replacement (SFHR)-mediated gene targeting, utilizing single- and double-stranded Small DNA Fragments (SDF), has previously been shown capable of effecting precise, specific genetic changes in the Cystic fibrosis transmembrane conductance regulators (CFTR), dystrophin, and zeocin genes [6-8]. These targeted genetic changes were demonstrated in a variety of cell types including human airway epithelial cells and mouse muscle cells. In this study, we evaluated the utility of SFHR-mediated gene targeting in organ specific stem cells with a view toward *ex vivo* stem cell gene therapy. Glass needle microinjection was utilized for delivery of SDF to human umbilical cord blood $Lin^{-}CD38^{-}$ stem/progenitor cells. Microinjection of primitive human blood stem/progenitor cells is a novel approach that allows delivery of SDF directly into the nucleus with minimal cell-to-cell variation in SDF dosage and with maintenance of progenitor or stem cell activity [9;10].

RESULTS

Microinjection-mediated delivery of β^S SDFs to human Lin^-CD38^- cord blood cells results in efficient β^A to β^S conversion

Genetic correction of rare, primitive blood stem cells is crucial to long-term treatment/cure of blood cell diseases. These studies focused on microinjection-mediated delivery of SDF to a very primitive population of human umbilical cord blood cells (i.e. Lin^-CD38^- cells). Lin^-CD38^- cells, 80-90% of which are $CD34^+$, are highly enriched for stem cells, in that approximately 1 in 600 $CD34^+CD38^-$ cells engraft NOD/SCID mice [11]. We have previously demonstrated that microinjected $CD34^+CD38^-$ cells have excellent survival and retain both their proliferative capacity as well as their ability to generate myeloid and erythroid progeny *in vitro*. Lin^-CD38^- cells were temporarily immobilized and microinjected with the 559 bp double stranded β^S SDF (Fig. 1).

Post-microinjection, cells were expanded in liquid culture and analyzed for the β^A to β^S conversion. Initial analysis entailed PCR amplification of chromosomal β-globin DNA in the progeny of microinjected Lin^-CD38^- cells (21-35 days after microinjection) utilizing primers that were outside the region of homology defined by the SDF (Fig.1; GD1 and GD2), followed by Dde I digestion. Successful β^A to β^S conversion results in the loss of a Dde I cleavage site within codon 6 of the human β-globin gene. A second analytical approach also involving PCR amplification of chromosomal β-globin DNA, but with only one primer outside the introduced SDF (Fig. 1; GCBGPy1 and GCBGPy2), followed by Pyrosequencing™. The pyrosequencing reaction employs a sequencing primer that anneals directly adjacent to the site of conversion to facilitate direct analysis of the target sequence (~ 8 nucleotides). This method permits assessment of the relative frequency of β^S vs. β^A alleles within the population of cells analyzed [12].

Fig. 2A depicts the results of representative Dde I digestion from experiments in which Lin^-CD38^- cells were injected with either Oregon Green dextran as control or β^S- SDF. These results indicate β^A to β^S conversion in some samples but not others. The sensitivity for detection of failure to digest at the Dde I site was approximately 5 - 10% (based on analysis of control standards having defined mixtures of cells having $\beta^A\beta^A$ or $\beta^S\beta^S$ genotype). Detectable β^A to β^S conversion was observed in 6 of 18 (33%) experimental samples originally microinjected with β^S- SDF. No conversion was observed in any of the non-microinjected or control microinjected samples (0 of 6 samples analyzed). Pyrosequencing results for the same experimental samples are presented in Fig 2B; the frequency of β^A to β^S conversion was determined for each experimental and control sample. Quantitative determination of allele conversion is demonstrated in Fig. 2C, in which Pyrosequencing accurately measured the frequency of β^S alleles (0 – 10%) in a background of β^A alleles. Pyrosequencing was capable of reliable detection of β^S alleles at frequencies of $\geq$ 1.5%.

Pyrosequencing detected β^A to β^S conversion in 13 of 18 (72%) experimental samples microinjected with β^S SDF. The conversion frequencies ranged from 1.65% to 8.6%. Each sample for which Dde I analysis showed β^A to β^S conversion was confirmed by Pyrosequencing (6 of 6). In addition, Pyrosequencing, likely due to its increased sensitivity, identified β^A to β^S

conversion in an additional 7 of 12 samples. Thus, this direct SNP sequencing method identified conversion in experiments that would otherwise have registered as negative. This observation also raises the possibility that other experimental samples had β^A to β^S conversion, but at a frequency too low for detection by either Dde I or Pyrosequencing.

β^A to β^S conversion in the progeny of engrafting Lin$^-$CD38$^-$ cells

Since β^A to β^S conversion occurred in a significant number of experimental samples, assessment of whether conversion occurred in true blood stem cells was assayed by engraftment in immune deficient mice. N/Sβ2mk/o mice were sub-lethally irradiated, prior to the delivery of the human Lin$^-$CD38$^-$ cells, to increase their engraftment in mouse bone marrow. Mice received, via tail vein injection, either irradiated mouse bone marrow (IMBM) alone (negative engraftment control; $2x10^4$ irradiated mouse bone marrow cells/mouse), a mixture of non-microinjected (NMI) Lin$^-$CD38$^-$ cells (1000-2500 cells per mouse) and irradiated mouse bone marrow cells, or a mixture of β^S-SDF microinjected (FMI) Lin$^-$CD38$^-$ cells (1000-2500 cells per mouse) and irradiated mouse bone marrow cells. IMBM was included in samples to decrease loss of Lin$^-$ CD38$^-$cells during the injection process.

The bone marrows of mice were analyzed for human β-globin sequences six weeks after the human cells were introduced. Human β-globin specific sequences were amplified with primers GD1/GD2 either from total bone marrow (TBM) or from bone marrow first depleted of mouse cells (MdBM). As summarized in Table 1, human β-globin sequences were identified in some, but not all recipient mice. A survey of the data revealed a number of samples in which human cells were only detectable after depletion of the mouse BM cells – e.g. samples 755H157 and 755H158. These findings are consistent with a low frequency of engraftment. The low frequency of human cells in mouse bone marrow was not unexpected since only 1000-2500 Lin$^-$ CD38$^-$ cells were transplanted, representing ~1-4 NOD/SCID repopulating cells [11;13;14]. There was no obvious adverse impact of microinjection on the ability of cells to engraft the N/Sβ2mk/o mice. These results were supported by the results from a larger number of transplanted N/Sβ2mk/o mice: 13 of 23 (57%) surviving mice transplanted with FMI Lin$^-$CD38$^-$ cells displayed human engraftment, compared with 3 of 10 (30%) transplanted with NMI Lin$^-$ CD38$^-$ cells (data not shown). Furthermore, results from transplanted NOD/SCID mice revealed that 4 of 17 (24%) surviving mice transplanted with FMI Lin$^-$CD38$^-$ cells displayed human engraftment, compared with 2 of 5 (40%) transplanted with NMI Lin$^-$CD38$^-$ cells (data not shown).

Conversion of β^A to β^S in mouse bone marrow samples was detected using the same approaches described for the detection of β^A to β^S conversion *in vitro*. Mouse bone marrow samples with detectable human cell engraftment were analyzed (GD1/GD2 amplifiable material; Table 1). Similar results were obtained from two independent PCR and Dde I digestion analyses. Control animals receiving no human cells did not demonstrate any evidence of human cell signal in isolated samples. Furthermore, control animals receiving non-microinjected Lin$^-$CD38$^-$ cells did not display any evidence for β-globin conversion. The presence of the β^S allele was clearly detected in 3 FMI mice (740H153, 755H157, 755H159) analyzed via Dde I digestion (Fig. 3, Table 1). Analysis of sample 740H153 demonstrated a mixture of the β^A and β^S Dde I RFLP patterns, evidence for a mixture of both alleles. Samples 755H157 and 755H159 showed only

Dde I RFLP pattern characteristic of the β^S allele, implying complete β^A to β^S conversion. Since Dde I analysis is only sensitive to ~10%, it was possible that up to 10% of alleles remained wild-type β^A. However, Pyrosequencing of the 755H159 sample revealed that 100% of the alleles had, in fact, been converted to β^S (Table 1). The remaining FMI mice showed only the wild-type, β^A allele. Sample 755H158 had no β^S sequences evident by Dde I digestion, a result confirmed by Pyrosequencing (Table 1).

The simplest explanation for the complete β^A to β^S conversion observed in samples 755H157 and 755H159 is that only a very small number (e.g. 1-2) of blood stem cells having both β-globin alleles converted from β^A to β^S were responsible for the engraftment in these mice. These results indicate that SFHR-mediated gene conversion may be a very efficient process in primitive blood stem/progenitor cells.

DISCUSSION

These results represent the first successful application of the SFHR technology to HSPCs capable of engrafting immune deficient mice. The utilization of targeted gene modification (genome editing) technology in HSPCs has previously been limited by several factors. First, electroporation or liposome-mediated transfection conditions allowing for efficient delivery of DNAs to primary HSPCs, without either significant loss of viability or stem cell function, have not yet been reported. Second, these non-viral methods yield extensive cell-to-cell variation in the number of molecules delivered per cell. Microinjection allows for the quantitative and nuclear-specific delivery of genetic material and thus avoids inefficiencies in uptake and transport to the nuclei associated with bulk transfection methods. Prior studies demonstrated that microinjection had no adverse effect on either proliferation or differentiation potential of blood progenitor cells [10]. In this study we extended this finding to stem cells, demonstrating equivalent engraftment of microinjected (vs. non-microinjected) HSPCs in both NOD/SCID and N/Sβ2mk/o mouse models.

These studies demonstrate SFHR-mediated gene conversion of β^A to β^S globin in normal cord blood derived cells. This report represents proof-of-principle studies in support of the ultimate goal of correcting the sickle cell anemia mutation in stem cells of sickle patients. The *in vitro* results were particularly encouraging with respect to the number of experimental samples showing conversion (13 of 18; 72%) as well as the frequencies of β^A to β^S conversion observed in these samples (1.6 - 8.6%). The progeny of the stem/progenitor cells injected in this study showed β-globin gene conversion at the DNA level 3-5 weeks (8-12 cell doublings) post-microinjection, consistent with previous *in vitro* studies demonstrating that SFHR-mediated nucleotide changes were passed on to subsequent generations [Goncz, et al. submitted] [6;7;15-17]. Although this report focuses exclusively on β^A to β^S conversion at the DNA level, we have obtained evidence that the β^A to β^S gene conversion is also reflected in β-globin mRNA transcribed in erythroid progeny [Goncz et al., submitted].

Engraftment of immune deficient mice with microinjected Lin^-CD38^- cells permitted evaluation of β^A to β^S gene conversion in the progeny of a very small number of transplanted engrafting cells. The demonstration of normal to sickle conversion in human blood cells capable of engrafting immune deficient mice is highly significant, as it strongly indicates that SFHR-mediated gene targeting in very primitive blood cells is possible. These results are also remarkable in that the normal to sickle conversion observed in mice 755H157 and 755H159 appears to be a homozygous conversion – that is a β^A to β^S conversion of both alleles. These data strongly suggest that SFHR-mediated gene targeting can be extremely robust in human blood cells. However, the process is clearly not 100% efficient since mouse 740H153 showed only partial conversion (β^A to β^S) in engrafting cells, and mice 740H155 (same experiment as 740H153) and 755H158 (same experiment as 755H157 and 755H159) showed no detectable β^A to β^S conversion. Based upon recent reports regarding the N/Sβ2mk/o model it is unclear whether the β^A to β^S conversion observed in our engraftment studies occurred in long-term SCID repopulating cell (SRC; as assayed in the NOD/SCID model) or shorter-term repopulating cell with broad lymphomyeloid differentiation potential[14;18]. Since the precise correlation between cell type read-out in immune deficient mouse models and reconstitution ability in humans (i.e.

the "true" human blood stem cell) is yet to be elucidated, we can conclude from our studies only that β^A to β^S conversion was observed in very primitive human blood cells.

Based on bone marrow transplantation studies, as few as 10% normal peripheral donor blood cells ($\beta^A\beta^A$) will dramatically improve the health of sickle patients [19-22]. Since under normal conditions, cells from sickle cell trait patients ($\beta^S\beta^A$) are functional, transplanted cells modified at only one allele may also have therapeutic benefit. Thus, the level of gene conversion achieved in this study could be of therapeutic benefit in patients with sickle cell disease, provided that the converted cells were maintained at significant frequency in the marrow of transplant recipients. Since the corrected mature red blood cells have prolonged survival advantage over sickle cells, it is possible that the percentage of corrected marrow stem cells that are required will be <10%.

Of obvious interest to us is the ability to deliver these SFHR molecules to blood stem/progenitor cells in bulk while providing for nuclear delivery of genome editing molecules without adverse effect on stem cell activity. It is likely that the most immediate application will be in those clinical situations where the corrected stem cells are at a selective advantage for survival and/or proliferation with respect to the endogenous, defective stem cells. This, for example, is believed to be the case for the successful therapy involving retrovirus-mediated delivery of the IL-2 receptor γ chain in the treatment of severe combined immune deficiency (SCID)[23]. Indeed, the successful demonstration of correction of a genetic mutation in blood stem cells and clinical success would open the door to application of this technology to numerous blood based diseases. As well, these technologies have potential wide-spread application to other classes of stem cells, including liver, pancreatic, and neuronal stem cells.

MATERIALS AND METHODS

Small DNA Fragment Production

Small DNA fragments (SDF) for sequence-specific modification of the human β-globin gene were designed to be homologous to the endogenous gene from upstream of exon 1 to downstream of exon 2 (Fig. 1). SFHR-mediated modification of wild-type β-globin (β^A) to sickle β-globin (β^S), is achieved by transfection of β^S-SDF, a region of the human β-globin sequence with a sickle cell disease "T" (versus the wild-type "A") at the 18th bp of the coding region of the gene. SDFs were constructed by polymerase chain reaction (PCR) amplification of DNA from either normal or sickle patients as previously described [6;24-26]. The 559-bp fragment was generated via PCR using the SC11/SC12 primer pair. The resulting β^S-SDF fragment was cloned into the pSK vector (pSK-SS) and sequenced. This vector was used as template for subsequent generation of preparative amounts of SDF (1 ng per 100 µl reaction). The SDFs were purified after amplification by precipitation in 70% ethanol. The quality and purity of SDF preparations can dramatically affect the efficiency of SFHR mediated repair. Not presented in this report are the results of several experiments utilizing a "poor" quality preparation of β^S SDF in which conversion was not observed.

Cells

Human hematopoietic stem/progenitor cells (HSPCs, Lin^-CD38^-) were isolated from cord blood [10]. Briefly, Lin^-CD38^- cells were purified from mononuclear cells (MNC) via negative selection with the StemSep system (Stem Cell Technologies Inc, Vancouver, Canada). The antibody cocktail removes cells expressing CD2, CD3, CD14, CD16, CD19, CD24, CD36, CD38, CD45RA, CD56, CD66b, and/or glycophorin A. Typically, 85-95% of the cell isolated (Lin^-CD38^-) are also $CD34^+$. Umbilical cord blood was obtained through an existing protocol from the University of Texas Medical Branch, Galveston, Department of Labor/Delivery (IRB protocol #95-282). In addition, MNC isolated from cord blood were purchased from Poietics (Cambrex, Walkersville, MD) and AllCells (Berkeley, CA). After purification, stem/progenitor cells were either frozen in Liquid N_2 or placed directly into culture in stem cell (SC) media containing Iscove's Modified Dulbecco's Media (IMDM, Gibco, Carlsbad, CA) supplemented with 1X BIT 9500 (50 mg/ml bovine serum albumin, 50 µg/ml bovine pancreatic insulin, 1 mg/ml human transferrin (Stem Cell Technologies)), 40 µg/ml low-density lipoprotein (LDL, Sigma), 50 mM HEPES buffer, pH 7.4 (Gibco), 20 ng/ml each of human thrombopoietin (TPO), flt-3 ligand, stem cell factor (SCF), and interleukin (IL)-6 (PeproTech Inc. (Rocky Hill, NJ, USA)). Cells were grown for a minimum of 18 hrs and a maximum of 72 hrs prior to microinjection.

Microinjection

Lin^-CD38^- cells were attached to Retronectin (RN, TaKaRa Biomedicals, Panvera, Madison, WI, USA) -coated dishes as previously described [10]. β^S-SDF (concentration of 2000-2500 double stranded molecules/fL) was delivered to attached cells by glass-needle mediated microinjection. The microinjection protocol was as previously described (10). On average 5000-10000 cells were successfully injected per session (total session lasting 8-10 hrs with 2 microinjectionists) with SDF. It is estimated that 0.5-2.0 fL of sample was injected per cell per successful injection. Routinely, an additional plate of 50-100 cells was microinjected with Oregon Green Dextran

(OGD, 70 kDa, Molecular Probes, Eugene, OR, USA, 0.3 mg/ml) to determine the success of the injection process. The success of injection of the HSPCs as determined by % viability (number of fluorescent cells/number of successfully injected cells x 100) typically ranges from 70-90%. Injected cells were detached by incubation with peptides [10]. Detached cells were transferred as total cell samples to wells of 48 well plates and cultured overnight in SC media prior to injection into immune deficient mice the following day.

For *in vitro* culture, microinjected cells were subsequently cultured in expansion (E) medium consisting of IMDM supplemented with 1X BIT 9500, 40 μg/ml LDL, 50 mM HEPES buffer, pH 7.4, 10^{-4} M β-mercaptoethanol, 50 ng/ml SCF, 10 ng/ml each of human IL-6 and macrophage colony-stimulating factor (M-CSF), 20 ng/ml each of IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF), 21 ng/ml granulocyte colony-stimulating factor(G-CSF), 3 U/ml human recombinant Erythropoietin (EPO, Stem Cell Technologies), and 100 μg/ml glutamine/penicillin/streptomycin for a minimum of 21 days to promote general expansion and differentiation of blood cells [27].

Immune Deficient Mouse Experiments

Modified Lin^-CD38^- cells were injected into NOD/LtSz-Prkdc^{scid} β2m^{tm1Unc}/J (N/Sβ2mk/o) or NOD/SCID mice. The N/Sβ2mk/o model allows for measure of the engraftment potential of smaller numbers of cells, as compared to previous immune deficient mouse models of human cell engraftment (e.g. the standard NOD/SCID mouse) because of the absence of β2 microglobulin, hemachromatosis, a lack of NK cells, T and B cells, and complement [28;29]. Colonies of N/Sβ2mk/o and NOD/SCID mice were established from breeder pairs received from Jackson Laboratories (Bar Harbor, ME, USA) and maintained under standard protocols.

Animals (6 wks old) were irradiated with 350-375 Rads, 24 hrs prior to intravenous tail vein injection with 100 μL of SC media containing 1000 to 2500 microinjected Lin^-CD38^- cells and $2x10^4$ irradiated mouse bone marrow cells. Lin^-CD38^- cells were supplemented with mouse bone marrow cells during the injection process to decrease loss of the Lin^-CD38^- cells. Engraftment was measured at 6 weeks following injection. Total bone marrow was collected from the leg bones of each mouse, subdivided, and either frozen until analysis, or further processed to enrich for engrafted human cells within the bone marrow. The majority of total bone marrow was subjected to enrichment procedures by magnetic depletion of mouse cells using the Mu-Hu kit (StemCell Technologies). Typically, ~80% of the mouse cells are removed, leaving a population highly enriched for human cells in the mouse-depleted samples (MdBM).

Isolation of Purified DNA

The presence of human β-globin and conversion from β^A to β^S-globin was ascertained by PCR amplification and RLFP analysis of purified cell DNA. Purified cell DNA was isolated by a modified Qiagen isolation method as follows. Pelleted cells were resuspended in 10mM TrisHCl (pH 7.8) buffer containing 50 mM EDTA, 5% SDS, and 2.9 mg/ml Proteinase K (Sigma), incubated for 10 min at 56°C, and diluted in 4 volumes of Qiagen AVL Buffer containing 26.6 mg/ml carrier RNA. Following 10 min at 20°C, DNA was precipitated with an equivalent volume of 100% ethanol and isolated using a QIAamp DNA Mini Kit Column (Qiagen Inc., Valencia, CA, USA). Purified DNA was eluted with 40 μL of 70°C warmed molecular biology grade H_20 (Sigma).

DNA Amplification

Two non-allele specific PCR amplification strategies were employed to determine the presence of β^S-globin in bone marrow isolated from mice following introduction of Lin^-CD38^- cells. Both primer sets amplify the human β-globin locus irrespective of genotype (i.e. both sickle and normal alleles will be amplified). Amplification with primers GD1 and GD2 (Fig. 1), both outside the region defined by the introduced β^S- SDF, insures that there will be no amplification of any residual β^S -SDF in the cells during PCR. Primers GCBGPy1 and Biotin-GCBGPy2 were designed to provide a highly efficient amplification of the β-globin gene around the 6^{th} codon (the site of the targeted genetic modification) (Fig. 1). Amplified material generated was employed in RFLP analysis with Dde1 digestion and Pyrosequencing™ analysis. No amplification of residual β^S- SDF was detected in control experiments when either GD1/GD2 or GCBGPy1/Biotin-GCBGPy2 primer sets were employed. Purified cell DNA (5 µl) was added to reaction mixture containing 1X FastTaq PCR buffer, 2 mM $MgCl_2$, 0.25 mM dNTP mix, 2.5 units FastStart Taq Polymerase (Roche Applied Science, Indianapolis, IN, USA), 0.25 µM Primer 1, 0.25 µM Primer 2, in a final volume of 50 µl and amplified via PCR.

Restriction Enzyme Analysis

Restriction digest of PCR amplicons performed with *DdeI* enzyme results in RFLP patterns that are distinct for β^A (201, 180, 158, 88, 89, 45, 37 and 3 bp) and β^S (381, 158, 88, 89, 45, 37, 3 bp) globin. The resultant digestion patterns were compared side by side with β^A and β^S - globin controls.

Pyrosequencing™ Analysis

Pyrosequencing™ analysis was performed according to manufactures protocol using the SNP Reagent Kit (Pyrosequencing AB, Westborough, MA, USA). Biotinylated PCR product was immobilized on streptavidin-coated Dynabeads M-280 Streptaviding (Dynal Biotech, Lake Success, NY, USA). The strands were separated by incubation for 1 minute in 0.5 M NaOH, and the biotinylated single strand was collected, washed, and transferred to wells containing 0.35 µM Sequencing Primer. A sequencing primer having the sequence 5'-GGTGCATCTGACTCT-3' was annealed to the Biotinylated single stranded PCR product by incubation at 80^0C for 2 min, prior to analysis with the PSQ96 instrument. The PSQ96 allelic discrimination software was utilized in analysis of pyrograms and determination of relative percentages of the "A" vs "T" at the sickle mutation site. Positive and negative controls were included in all Pyrosequencing™ runs.

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FIGURE LEGENDS

Figure 1: Schematic representation of human β-globin gene and amplification primers used in analysis

Figure 2: Successful modification of genomic β-globin by SFHR.
Samples were analyzed by both DdeI digestion (panel Aii) and Pyrosequencing (panel B). DNA isolated from progeny of cells microinjected with either OGD (lane 1, E694-1) or β^S SDF (lanes 2 -6; E698-2, E696-3, E684-2, E694-4, and E682-1, respectively), or control cells, Lin^-CD38^- cells (lane 4), SC-1 cells (lane 7) or known mixtures of Lin^-CD38^- and SC-1 cells (lanes 5 and 6, mixtures of 5% SC-1/95% Lin^- and 10% SC-1/90% Lin^-, respectively) were amplified by PCR with GD1/GD2 (panel Ai) and digested with Dde1 (pane Aii). B) Isolated DNA from samples corresponding to samples analyzed in panel A were amplified using primers GCBGPy1/GCBGPy2 and Pyrosequenced. C) Quantitative Determination of allele frequency conversion by Pyrosequencing. DNA containing known amounts of normal and sickle β-globin was amplified using primers GCBGPy1/GCBGPy2 and subjected to Pyrosequencing. Representative pyrograms from DNA samples containing 100% β^A (i); 98% β^A:2% β^S (ii); 95% β^A:5% β^S (iii); 90% β^A:10% β^S (iv); and 100% β^S (v) are shown. Pyrograms are read from left to right with peaks indicating the presence of the specific nucleotide listed on the X-axis.

Figure 3: Dde1 restriction enzyme digestion of GD1/GD2 amplified material from mouse samples. GD1/GD2 amplified material was digested with Dde1, isolated on a 2.5% NuSieve gel and stained with SyBr Green. Lane 1, water control; lane 2, DNA ladder (100 bp, NEB); lanes 3, 5, 6, 7, 8 represent digests of material amplified from mouse-depleted bone marrow samples 740H153, 740H155, 755H157, 755H158, and 755H159, respectively; lane 4 represents digested material amplified from the total bone marrow of mouse 740H155. Dde1 digested material amplified from Lin^-CD38^- human cells (normal control) and SC-1 cells (sickle control) in lanes 9 and 10, respectively.

Table 1: Summary of results obtained in experiments involving the delivery of SFHR fragment microinjected human Lin^-CD38^- to N/Sβ2mk/o.

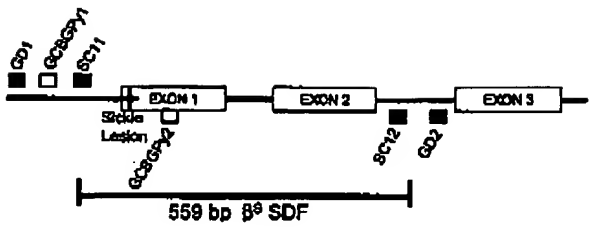
Mouse	Cells Injected into Mouse	DNA From	GD1/GD2 Amplification	β-globin Species A= normal; S = sickle	Pyrosequencing™ (%A (normal):%T (sickle)
625B1	NMI Lin^-CD38^- [a]	MdBM [c]	+	A	
625B2	NMI Lin^-CD38^-	MdBM	+	A	
625C1	FMI Lin^-CD38^- [b]	MdBM	+	A	
625C2	FMI Lin^-CD38^-	MdBM	+	A	
740H150	NMI Lin^-CD38^-	TBM [d]	-		
740H151	NMI Lin^-CD38^-	TBM	-		
740H153	FMI Lin^-CD38^-	TBM	-		
		MdBM	+	A + S	
740H154	FMI Lin^-CD38^-	TBM	-		
		MdBM	-		
740H155	FMI Lin^-CD38^-	TBM	+	A	
		MdBM	+	A	
755H157	FMI Lin^-CD38^-	TBM	-		
		MdBM	+	S	
755H158	FMI Lin^-CD38^-	TBM	-		
		MdBM	+	A	100:0
755H159	FMI Lin^-CD38^-	TBM	-		
		MdBM	+	S	0:100
755H161	IMBM only	TBM	-		-

[a] Non-Microinjected Lin^-CD38^- cells

[b] Fragment Microinjected Lin^-CD38^- cells (Lin^-CD38^- cells microinjected with β^S SDF)

[c] Mouse-depleted bone marrow

[d] Total Bone Marrow


GD1
GCSGFP1
SC11
EXON 1
EXON 2
EXON 3
Sickle
Lesion
GCSGFP2
SC12
GD2
559 bp β^S SDF

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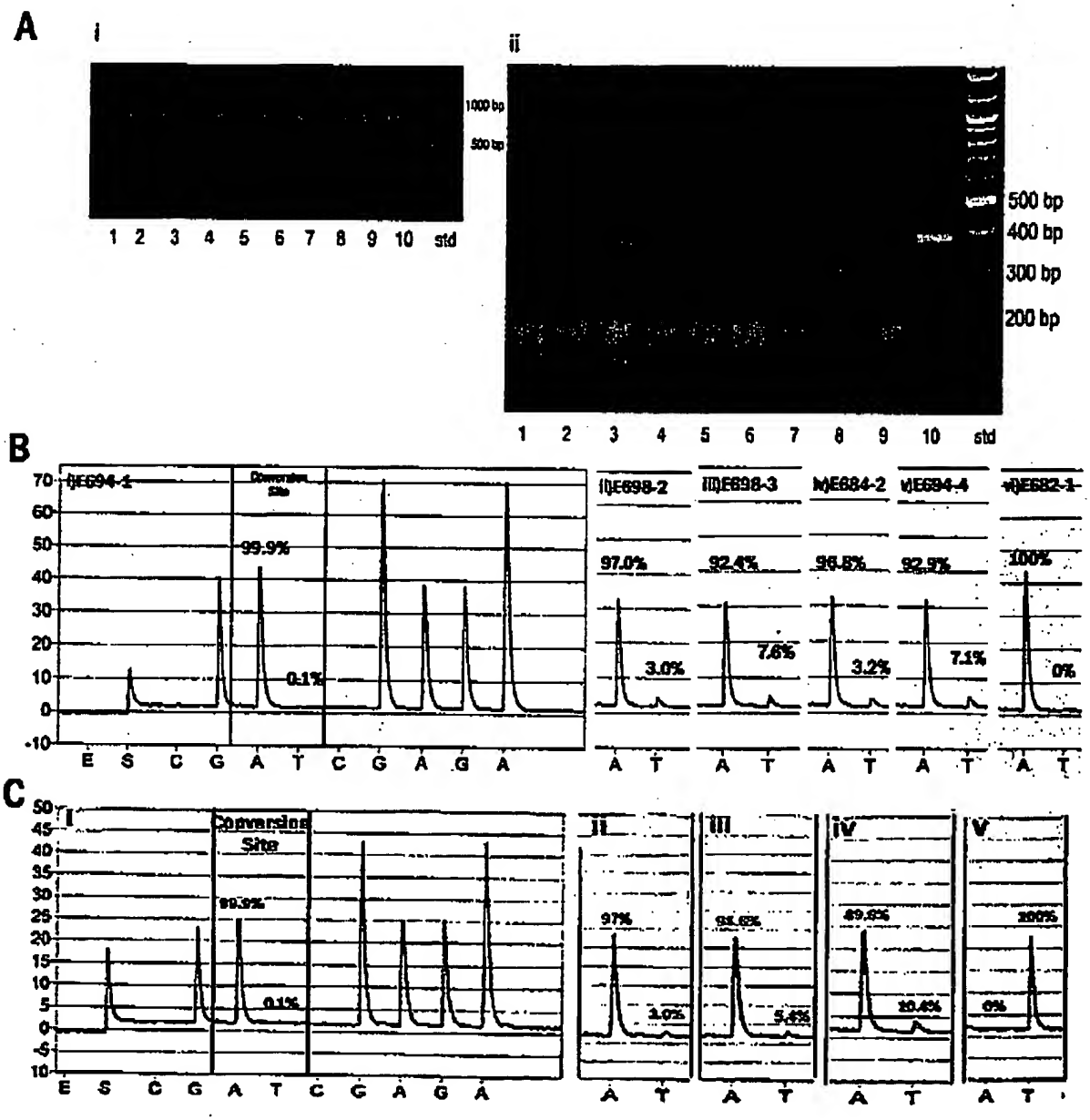

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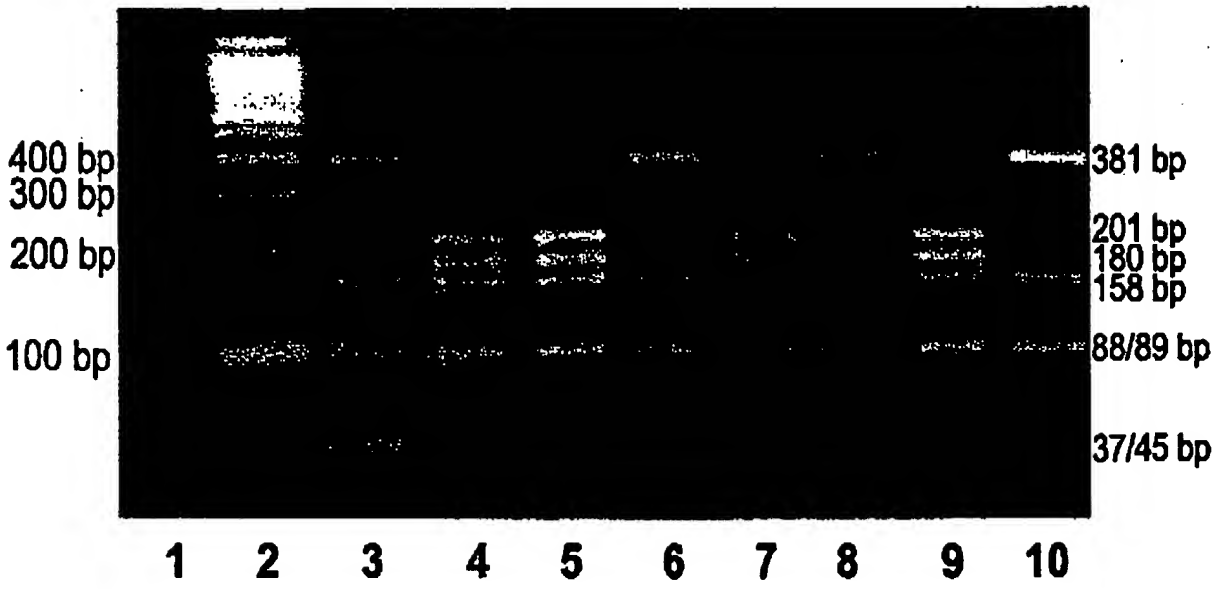

EXHIBIT B

MODIFICATION OF GENOMIC ß-GLOBIN SEQUENCES IN HUMAN HEMATOPOIETIC STEM/PROGENITOR CELLS BY SMALL DNA FRAGMENTS: IMPLICATIONS FOR *EX VIVO* GENE THERAPY

Running Title: SFHR-mediated modification of ß-globin sequences

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Key Words: microinjection, SFHR, gene targeting, sickle cell

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The direct modification of genomic sequences has been demonstrated by oligonucleotide gene targeting strategies. Small fragment homologous replacement (SFHR) -mediated gene targeting strategy utilizes small DNA fragments (SDF) and has been shown to simultaneously modify up to 4 bp of DNA sequence in a site-specific manner *in vitro* and *in vivo*. SFHR can, therefore, potentially be used to correct or modify specific disease genes for which the sequence is known. Sickle cell disease (SCD) is a recessive inherited disorder that results from a single-base pair transversion (A → T) in the human ß-globin gene. In this study, we investigated the feasibility and efficacy of using SFHR to modify ß-globin sequences in normal human hematopoietic stem/progenitor cells (HSPC). SDF (559 bp) comprised of sickle ß ($ß^S$) -globin sequence were microinjected into the nucleus of individual $lin^-/CD38^-$ cells at concentrations between 250 - 10,000 SDF per cell in 70 separate experiments. Site-specific conversion ($ß^A$- → $ß^S$-globin) was observed in ~50% of the experiments as determined by DNA and RNA analysis. The frequency of allele conversion in each experiment was between 1-10%. The results demonstrate that SFHR could be used as a gene therapy for SCD through SFHR-mediated correction of the $ß^S$-globin sequence in a patient's HSPC followed by autologous transplantation.

Introduction

Sickle cell disease (SCD) results from a single-base pair transversion (A → T) in the 6th codon of the human ß-globin gene. SCD afflicts 1 in 200 to 1 in 400 blacks in the US and as many as 1 in 64 blacks in Africa.[1] In some areas of Africa the frequency may be as high as 1 in 44 to 1 in 25.[2] Clinical features of the disease include vaso-occlusive complications, episodes of pain, stroke, osteonecrosis and retinal degeneration among others.[3] Currently, treatment for SCD includes blood transfusion, bone marrow transplantation, and exposure to hemoglobin F-inducing agents such as 5-azacytidine, butyrate and hydroxyurea.[4-6] However, there are a number of complications with these treatments including transfusion-induced alloimmunization and the potential mutagenic, carcinogenic and/or leukemogenic effects of hemoglobin F-inducing agents.[5-8]

As an alternative to pharmacological or allogenic transplantation approaches, genetically based therapies show promise for the treatment and in theory, cure of SCD. For example, the introduction of a normal or wild-type (wt) copy of the ß-globin gene ($ß^A$) into hematopoietic stem/progenitor cells (HSPC) of an SCD patient followed by autologous transplantation may be effective[9] and could circumvent many problems associated with allogenic transplantation.[10] Ongoing experiments have demonstrated that introducing the coding sequence for $ß^A$-globin into HSPC is possible through viral-based expression vectors including retrovirus,[11,12] lentivirus,[13] foamy virus[14] and adeno-associated virus.[15,16] There have; however, been a number of issues that have arisen with these viral vector systems that may undermine their overall effectiveness. Limitations in viral strategies include a lack of long-term expression and position effects on gene expression due to the site of viral integration, cell-surface presentation of viral antigens, and inefficient viral transduction of target cell populations. In addition, a recent gene therapy clinical trial result indicated that insertional mutagenesis by a DNA vector might have oncogenic outcomes.[17]

An alternative gene therapy strategy for SCD would be the direct correction of mutant $ß^S$-globin gene sequence within the genomic DNA of HSPC followed by autologous transplantation of modified/corrected cells. The advantages to such a strategy over strategies that involve the introduction of $ß^A$-globin coding alone are that the integrity of $ß^A$-globin gene expression is retained and no exogenous non-human nucleic acid sequences (i.e. viral vector or bacterial) are introduced into the genomic DNA. Transcription of the ß-globin gene thus remains under the regulation of endogenous cell-specific elements and any immune response mediated by viral and bacterial sequence and/or protein elements will be mitigated.

Direct correction or site-specific modification of DNA sequences has been demonstrated in a variety of systems by oligonucleotide gene targeting strategies (see reviews[18-20]). Specifically, site-specific modification of the ß-globin gene has been accomplished by both chimeric RNA/DNA oligonucleotides[21,22] and small fragment homologous replacement (SFHR).[23] SFHR has shown promise for the gene therapy of other inherited disease including cystic fibrosis (CF)[24-27] and Duchene's Muscular Dystrophy (DMD).[28] This is, in large part, due to its flexibility with regard to modification of different DNA targets.[25,27-32] SFHR involves the introduction of small (~500 bp) DNA fragments (SDF) that are homologous to the target sequence except for the desired modification(s). The SDF can be readily generated by PCR amplification without the need for specialized synthesis protocols. The modification of up to 4 bp simultaneously has been demonstrated by SFHR whereas chimeric RNA/DNA oligonucleotides appear to be

limited to one bp. In addition, SFHR has been successfully employed *in vitro* as well as *in vivo* and has resulted in the modification of transcribed and non-transcribed genes in a variety of cell types.

In this study, we investigated whether SFHR has the potential as a gene therapy for SCD. SDF (559 bp) with β^S-globin sequence and a unique restriction site (Afl II) were introduced by microinjection into normal human HSPC ($lin^-/CD38^-$ isolated from human umbilical cord blood). Modification of genomic β^A-globin sequences and subsequent expression of β^S-globin was determined by restriction fragment length polymorphism (RFLP) analysis of PCR amplification of DNA and mRNA-derived cDNA as well as through cloning and sequence analysis.

Results

Microinjection was found to be an effective and well-tolerated method for delivery of SDF to human HSPC. Based on Oregon Green Dextran (OGD) fluorescence in the nucleus, up to 91% of microinjections were successful and the average number of successful injections per experiment was consistent for both OGD and SDF microinjected HSPC (Table 1). Of the successful injections, as determined by nuclear retention of OGD and visualization of cell viability, up to 80% of the HSPC were viable 2 h post microinjection (average ± S.D. = 56 ± 16%). In SDF experiments, a greater proportion of HSPC were microinjected than in experiments where OGD was injected alone however, the relative number of successful injections remained similar (Table 1). The growth rate of OGD microinjected HSPC, SDF microinjected HSPC and non-microinjected control HSPC were similar: an average of 1 cell doubling every 1.5 days.

SFHR-mediated conversion of β^A- to β^S-globin was observed in 42% of experiments overall (Table 1). The time when cells were harvested after SDF delivery ranged from 9 to 49 days. This is equivalent to a 4-32 fold population doubling before cells were harvested and implies that SFHR-mediated modification was stably inherited at the chromosomal level. Successful modification of genomic sequences was routinely determined on PCR-generated DNA amplicons by RFLP analysis (Figure 2) or Pyrosequencing.[33] All the experiments demonstrating successful SFHR-mediated modification detected both the SFHR-derived β^S-globin and endogenous β^A-globin sequence. This result was expected since only a defined number of cells were microinjected in each experiment (Table 1). Furthermore, of the injected cells, only a fraction of the cells are likely to undergo successful SFHR-mediated modification. For example, in one experiment, E442-1, 217 out of 250 cells were microinjected with SDF at a concentration of 2000 fragments/fL (Figure 2, lane 3). Of the microinjected cells, only 140 of the cells successfully received SDF, and based on nuclear OGD retention, only 78, or 70%, survived microinjection to proliferate and differentiate in culture. These SDF transfected cells were grown with the 33 cells that were not microinjected for a period of 24 days before harvest. RFLP analysis of 23 individual bacterial colonies of cloned PCR amplicons from experiment E442-1 showed that 3, or 13%, of the amplicons contained β^S-globin sequence while none showed the presence of the Afl II site. This implies that modification could have occurred on one allele in 14 of the 78 cells that survived microinjection. Qualitative RFLP assessment of the proportion of modified alleles showed that between 1 and 10% of the β^A-globin alleles were modified to β^S-globin sequence in all of the experimental samples showing conversion.

A correlation was observed between successful β^A-globin sequence modification and the number of SDF molecules delivered. The most consistent modification (~51% of experiments showing conversion) occurred when SDF was delivered at a concentration of 2000 fragments/fL (Table 1). SDF delivered at 1000 fragments/fL resulted in 44% of experiments showing conversion whereas experiments with 500 fragments/fL showed no conversion (Table 1). A small number of experiments (n=3) were performed with SDF at concentrations between 3000-5000 fragments/fL. No conversion was observed in these experiments. Experiments also revealed that the quality of the SDF preparation played a crucial role in targeted replacement. While some preparations of SDF resulted in efficient conversion, other preparations used in parallel experiments yielded no detectable conversion. What differentiates these SDF preparations is not yet known. It has; however, been shown that DNA purity is a factor in the transfection efficiency of plasmid DNA.[34] The proportion of microinjected cells to number of cells plated (Table 1) also correlates with SFHR-mediated conversion in that this ratio is lower in successful experiments. Typically the number of injected cells is a reflection of the factors influencing the microinjection process including overall cell health, sample/DNA quality, and needle quality. Ideally, when all these factors are optimal, the number of injected cells to total cells is lower.

There was no correlation observed between the number of microinjected cells and the frequency of allele conversion. This result can be attributed, in part, to the variable growth rates of individual HSPC within the population[35] and, as such, the proportion of modified alleles, analyzed after several days of culture, differs from the initial number of modified alleles. Thus, unless individual microinjected cells are grown separately, it is difficult to determine if the fraction of modified alleles detected can be attributed to many modified cells that grew at the same rate or to a few modified cells that grew rapidly. Nevertheless, a calculation of the range of allele modification frequency for SFHR can be determined. For example, in one experiment, only 18 HSPC were viable after microinjection. They were grown in a bulk population along with 959 noninjected cells. RFLP analysis of the PCR amplification product from this experiment, although not strictly quantitative, indicated that ~1-5% of the total β^A-globin alleles were successfully modified to β^S-globin. If both β^A-globin alleles were successfully modified in all the microinjected cells (100% allele modification frequency) and all the cells in the population grew at the same rate, then an overall modification frequency would be 1.8% (number of modified alleles =36/total alleles =1954). Alternatively, if only one allele in a single cell was modified and the microinjected cells overran the population, then an overall modification frequency would be 2.7% (number of modified alleles = 1/total alleles = 36).

Successful modification of endogenous β^A-globin alleles resulted in expression of β^S-globin mRNA after microinjected cells were placed in culture conditions designed to promote erythropoiesis. Expression was confirmed by RFLP analysis of the amplicon from non-allele specific PCR amplification of mRNA-derived cDNA (Figure 2). Cloning of the PCR amplicon from one experiment indicated that 10% of the ß-globin mRNAs were β^S-globin (8 out of 80 clones analyzed) 49 days after microinjection. The frequency of conversion at the DNA level was determined to be 11.1% by Pyrosequencing[33]. These studies demonstrate that SFHR-mediated modification of the ß-globin allele in human HSPC following microinjection of SDF is stable and results in expression of the targeted gene.

Discussion

The results presented demonstrate that SFHR can be used to modify a significant fraction (1-10%) of the ß-globin alleles in a population of HSPC and that modification of genomic DNA leads to expression of ß-globin RNA. Several factors strongly argue that the $ß^A$- to $ß^S$-globin replacement observed in these experiments was not simply a spurious PCR artifact generated during the analysis. First, the PCR primers utilized for analysis are completely outside the region of homology defined by the SDF. Second, the SDF copy number at time of analysis was significantly less than that required to cause spurious amplification. For example, if 5000 fragments are injected into a single cell, after 11 doublings there would be ~2000 cells. Thus, the average number of fragments per cell is approximately 2.4. Previous studies addressing the potential of PCR generated false positives demonstrated that spurious amplification is not detected even when the number of SDF is $1x10^6$ per cell[24]. Third, the failure to detect the Afl II site, present in the original SDF, argues against the presence of original SDF at time of analysis. The specificity of the SFHR-mediated conversion, as opposed to random integration of SDF, is also supported by our analysis. Analysis of microinjected samples not showing conversion (but microinjected with SDF) did not produce detectable $ß^S$-globin sequence within the genome, as analyzed by allele-specific PCR amplification using $ß^S$-globin allele-specific primers located within the region defined by the SDF.

The mechanisms underlying SFHR-mediated modification have yet to be elucidated. However, these results suggest possible mechanisms. It appears that the complete SDF sequence does not incorporate into the endogenous genomic DNA, suggesting that a possible preference of a specific region of the input SDF occurs during SFHR-mediated modification. The silent mutation engineered into exon 2 of the $ß^S$-globin SDF (Afl II site) has yet to be detected in the analysis irrespective of whether experimental samples demonstrated targeted $ß^A$- to $ß^S$-globin replacement. Unlike previous SFHR studies in which the unique restriction site was engineered into the SDF within the same exon as the target sequence (100 bp upstream of the primary modification sequence),[24, 25] the Afl II site in the $ß^S$-globin SDF was located ~350 bp downstream of the sickle mutation target and in a different exon (exon 2). This observation suggests that there may be sequence- and/or region- specific preferences, either within chromosomal DNA or within the SDF, that affect the efficiency of SFHR-mediated modification. Another mechanistic indicator results from one experiment in which microinjected cells were isolated by limiting dilution and expanded. In this experiment, cells were microinjected with ~5000 fragments per cell. Analysis of a clone from this experiment by RFLP analysis of the PCR amplification product indicated that ~10% of the $ß^A$-globin alleles were successfully converted to the $ß^S$-globin sequence. Since this sample originated from a single microinjected cell, the result implies that the SFHR-mediated modification was either resolved after cell division or occurred to only one DNA strand (sense or antisense) of an allele. This might suggest that possible partial incorporation of the SDF occurs through strand invasion.[36]

These results demonstrate that SFHR-mediated modification of the ß-globin allele is possible in HSPC. Further studies indicate that such modified cells can engraft in mouse model of human blood stem cell engraftment.[33] However, the question still remains if these studies provide sufficient evidence as to suggest a role for SFHR-mediated targeted gene modification in *ex vivo* gene therapy for SCD. Theoretically, one pluripotent cell can reconstitute the hematopoietic system.[37] Unfortunately, this cell remains to be identified. It was recently

shown that transplantation of CD34 selected and *ex vivo* expanded cells from a single cord blood aliquot is sufficient for patient survival.[38] In these studies, children (average weight of 20 kg) were infused with 21.1 x 10^4 $CD34^+$ cells per kg. The cells were delivered in 2 fractions for complete hematopoietic reconstitution. Immediately after a high dose therapy regimen, patients received 40 or 60% of the isolated $CD34^+$ cells for long-term reconstitution. The remaining cells were expanded in culture for 10 days in media containing stem cell factor, granulocyte colony stimulating factor and megakaryocyte growth and differentiation factor to provide enough cells for short-term reconstitution. Given that the median expansion *ex vivo* was a factor of four, ~3.0 x 10^4 $CD34^+$ cells per kg were required for long-term reconstitution. It has been proposed that only 5-20% of ß-globin producing cells would be needed to produce enough $ß^A$-globin to restore a normal phenotype in SCD patients.[39] In other words, restoration of a normal phenotype may be possible with an autologous transplantation of between 1.5 - 6 x 10^3 corrected HSPC per kg (a 20 kg child would require 3-12 x 10^4 cells for transplant). Currently, it is possible to microinject 1 x 10^4 HSPC with SDF in an 8 h period (personal communication N. Prokopishyn). The results presented here demonstrate that the minimum level of this requirement is within reach. The development of a more efficient delivery system (e.g. more microinjection workstations), improved SFHR-mediated modification, enhancement of the expansion potential of HSPC for long term repopulation, or the identification of pluripotent cells, will make *ex vivo* SFHR-based therapy a reality.

Materials and Methods

SDF

The SDF used in these studies was generated by PCR amplification and consisted of exons 1 and 2 of the $ß^S$-globin gene as well as 29 bp of the 5'-sequence upstream from exon 1 and 34 bp of the intron sequence downstream from exon 2. The SDF sequence is homologous to the $ß^A$-globin gene except for the A→ T transversion at codon 6 (the sickle mutation) and a silent bp exchange in the 81st codon that results in a unique Afl II (cttaag) restriction site. The Afl II site was created by megaprimer technique to exchange the final C → T.[40] Preparative amounts of the 559 bp SDF were generated with primer pair SC11 (5'-aaagtcagggcagagccatcta-3') and SC12 (5'-gggaaagaaaacatcaagggtc-3') using, as a template, a cloning vector (pSK, Invitrogen) that contains a sequenced copy of the 559 bp SDF. The PCR conditions were as follows: initial denaturation, 95°C/2 min followed by 25 cycles of denaturation, 95°C/30 sec, annealing, 60-65°C/30 sec, extension, 72°C/1 min with a 7 min extension in the final cycle. The PCR mixture contained: 200 µM dNTPs, 200 nM each primer, 1X Taq Polymerase Buffer (Perkin Elmer), 2 mM $MgCl_2$ and 0.5 units of Taq (Perkin Elmer) in a 100 µl reaction. The SDF were purified by ammonium acetate and ethanol precipitation followed by resuspension in water at a final concentration of 1 µg/µl.

Cells

Human HSPC (lin^-CD38^-) were isolated from human cord blood as previously described.[41] Umbilical cord blood was obtained through an existing protocol from the University of Texas Medical Branch, Galveston, Department of Labor/Delivery (IRB protocol #95-282). Typically, 85-95% of the cells isolated in this way (Lin^-CD38^-) are also $CD34^+$. After purification of HSPC, the isolated cells were either frozen in liquid nitrogen or placed directly into culture in stem cell (SC) media containing Iscove's Modified Dulbecco's Media (IMDM, Gibco/Invitrogen, Carlsbad, CA) supplemented with 1 x BIT 9500 (50 mg/ml bovine serum albumin, 50 µg/ml bovine pancreatic insulin, 1 mg/ml human transferrin (Stem Cell Technologies)), 40 µg/ml low-

density lipoprotein (LDL, Sigma), 50 mM HEPES buffer, pH 7.4 (Gibco), 20 ng/ml each of thrombopoietin (TPO), flt-3 ligand, stem cell factor (SCF), and human interleukin (IL)-6 (PeproTech Inc. (Rocky Hill, NJ, USA)). Cells were grown for a minimum of 18 h and a maximum of 72 h prior to microinjection.

Microinjection

For microinjection, HSPC were attached to retronectin (RN, 50 ng/µl CH-296 fibronectin fragment (TaKaRa Biomedicals, Panvera, Madison, WI, USA)) coated 35 mm plastic dishes by incubation of the cells on dishes for 45 minutes at 37°C as previously described[41] Approximately 300-500 cells were attached to plates within a confined area of 2.5 mm defined by a cloning ring. SDF (concentration of 500-3500 copies/fL) were introduced into attached cells by glass-needle mediated microinjection. Microinjection needles (outer tip diameter 0.17-0.30 µm) were pulled from 10 cm borosilicate capillaries with a 1.2 mm outer/0.94 mm inner diameter using a Flaming/Brown Micropipette Puller Model P-97 (Sutter Instrument, Novato, CA, USA). Cells were microinjected under an Olympus IX70 inverted microscope equipped with an electronically interfaced movable stage (Prior Scientific) and the electronically interfaced Eppendorf Micromanipulator (Model 5171) and Transjector (Model 5246). It is estimated that 0.5 - 2.0 fL of sample was injected per cell per successful injection. In addition to experimental dishes, control dishes in which 50-100 cells were microinjected with 0.3 mg/ml Oregon Green Dextran (OGD, 70 kDa, Molecular Probes, Eugene, OR) were set-up to visualize and establish the success of the injection process. The number of cells that retain nuclear fluorescence divided by the number of successful injections is considered as the viability of an experiment.

All of the cells within the cloning ring were detached by incubation with a mixture of peptides (consisting of fibronectin CS-1 fragment (0.42 mg/ml), Phenylac-Leu-Asp-Phe-D-Pro-NH2 (VLA-4i, 1.0 mg/ml, and H-Arg-Gly-Asp-Ser-OH (RGDS, 1.0 mg/ml) (Bachem BioScience, Torrance, CA, USA) for 15 min at 37°C. Detached cells were transferred in bulk into separate wells of a 48-well plate or subject to limiting dilution and transferred individually into separate wells of a 96-well plate. The cells were grown overnight in SC media. Cells were subsequently cultured in expansion (E) medium consisting of IMDM supplemented with 1x BIT 9500, 40 µg/ml LDL, 50 mM HEPES buffer, pH 7.4, 10 µM ß-mercaptoethanol, 50 ng/ml SCF, 10 ng/ml each of human IL-6 and macrophage colony-stimulating factor (M-CSF), 20 ng/ml each of IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF), 21 ng/ml granulocyte colony-stimulating factor (G-CSF), 3 U/ml human recombinant Erythropoietin (EPO, Stem Cell Technologies), and 100 µg/ml glutamine/penicillin/streptomycin for a minimum of 21 days to promote general expansion and differentiation of blood cells [42] or IMDM supplemented with 1x BIT 9500, 40 µg/ml LDL, 50 mM HEPES buffer, pH 7.4, 10 µM ß-mercaptoethanol, 1 pg/ml GM-CSF, 21 ng/ml 0.01 U/ml IL-3, 10 U/ml human recombinant EPO, and 100 µg/ml glutamine/penicillin/streptomycin.[43] Cells were grown for different periods, harvested and stored in microfuge tubes in aliquots of ~1 x 10^{3-6} cells at -80°C.

DNA and RNA isolation

DNA or RNA was isolated from frozen cell pellets containing >1 x 10^4 cells by DNAzol or Trizol (Gibco BRL), respectively according to manufacturer's directions. DNA was released from cell pellets (≤1 x 10^4) by incubation in cell lysis buffer (5mM Tris-HCl, pH 8.5, 250 µg/ml proteinase K, 0.45% Nonidet P-40, 0.45% Tween 20) at 56 °C for 1 h followed be proteinase K inactivation by a 10 min incubation at 95°C. Samples were stored on ice or –20 °C before PCR

amplification. Isolated RNA was resuspended in DEPC water and subject to reverse-transcription (RT) to produce mRNA-derived cDNA using SuperScript II (Invitrogen) according to manufacturer's instructions.

PCR amplification of DNA and mRNA-derived cDNA

Non-allele specific amplification of DNA was performed using primer pair SC3 (5'-ccctgtggagccacaccctagggt-3') and SC4 (5'-aacgatcctgagacttccacact-3'). These primers are located outside the region of homology defined by the SDF and produce a 742 bp amplicon for both $ß^A$- and $ß^S$-globin genomic sequences. Non-allele specific amplification of mRNA-derived cDNA was performed using primer pair SC5 (5'-acatttgcttctgacacaactgtg-3') and SC14 (5'-actggtggggtgaattctttgc-3') that produce a 430 bp amplicon for both $ß^A$- and $ß^S$-globin. Given the structure of the ß-globin gene and the location of the $ß^S$-lesion, only one primer, SC14, is located outside the region of homology defined by the SDF, however, it is located in exon 3 and will produce a 1410 bp amplicon with a DNA template. Allele-specific PCR amplification of both DNA and mRNA-derived cDNA was performed using the sense primer SC9A (5'-accatggtgcacctgactcctga-3') for $ß^A$-globin or SC9S (5'- accatggtgcacctgactcctgt-3') for $ß^S$-globin in conjunction with antisense primer SC4 (DNA template, 593 bp) or SC14 (mRNA-derived cDNA template, 382 bp). PCR amplification conditions are given above, except that amplification was performed for cycles.

Restriction Enzyme Analysis

Because $ß^A$-globin sequence has a Dde I site (ctgag) which is absent in $ß^S$-globin sequence (ctgtg), RFLP analysis of amplicons can be performed on non-allele specific PCR amplification products from DNA and mRNA-derived cDNA with Dde I enzyme. The resultant RFLP patterns are specific for $ß^A$- and $ß^S$-globin sequences. Amplicons were digested after PCR amplification with enzyme buffer and 10 U of Dde I (New England BioLabs, Beverly, MA, USA) per 200-300 ng PCR amplicon. Restriction digestions were separated by gel electrophoresis using NuSieve 3:1 agarose (BioWhittaker, Walkersville, MD). Control digestions included DNA isolated from normal and sickle (SC1, ATCC) cells.

Cloning of PCR amplicons and RFLP analysis

PCR amplicons from non-allele specific amplification of both DNA (SC3/SC4) and mRNA-derived cDNA (SC5/SC14) were cloned into the TA cloning system as per manufacturer's instructions (InVitrogen). Individual clones were analyzed as follows. Briefly, separate bacterial colonies were resuspended in 100 µl of LB (100 µg/ml ampicillin). Suspensions were grown for 1 h (37°C, 225 rpm). Bacteria were lysed by addition of 10 µl of the suspension to 30 µl of water and heating to 94°C for 10 min. PCR amplification was performed directly on the lysed bacteria after the addition of 60 µl of PCR solution as described above. The PCR amplicons were then digested with Dde I and run on a 3% NuSieve gel as described above.

Table 1: Critical parameters for microinjection experiments

Experiment (n=number)	Conversion (%)	SDF (# frag/fl)	HSPC (n ± S.D.)		
			Cells Plated	Cells Injected	Successful Injections
OGD (45)	NA	NA	588 ± 266	99 ± 26	58 ± 16
SDF (72)	42%	1590 ± 766	578 ± 243	377 ± 217	261 ± 163
SDF (30)	100%	1617 ± 486	532 ± 269	262 ± 177	179 ± 135
SDF (42)	0%	1571 ± 921	611 ± 219	459 ± 207	322 ± 154
SDF (18)	100%	2000	489 ± 236	332 ± 195	225 ± 156
SDF (17)	0%	2000	600 ± 185	524 ± 183	360 ± 146
SDF (12)	100%	1000	596 ± 312	157 ± 60	110 ± 48
SDF (15)	0%	1000	714 ± 250	480 ± 227	333 ± 174
SDF (7)	0%	500	410 ± 129	247 ± 92	188 ± 68

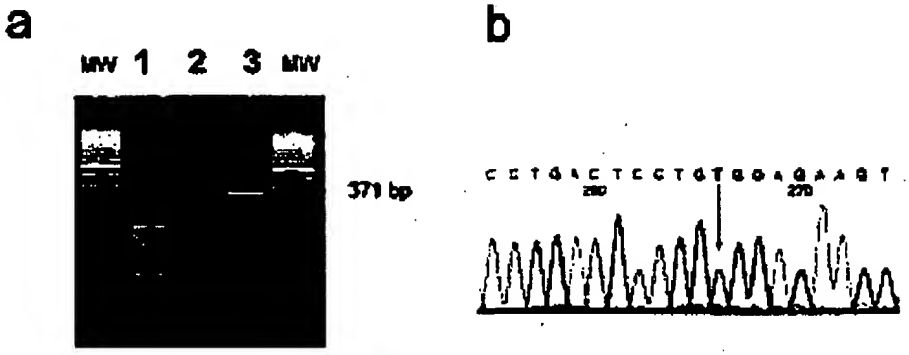

Figure 1: Representative examples of different methods that were used to confirm SFHR-mediated modification of genomic ß^A-globin sequences.
a) Dde I digest of PCR amplicons from non-allele specific PCR amplification of isolated DNA. The RFLP patterns for ß^A- and ß^S-globin are shown in lane 2 and 3, respectively. Lane 1 shows the RFLP pattern from experiment E442-1. Successful SFHR-mediated modification of the ß^A-globin sequence to ß^S-globin is indicated by the loss of a Dde I site shown by the 371 bp band. MW lane is an 100 bp MW ladder. b) Sequence analysis from pSK-cloning of SC3/SC4 PCR amplicon products from experiment E442-1. The sequence confirms the A → T conversion mediated by the SDF.

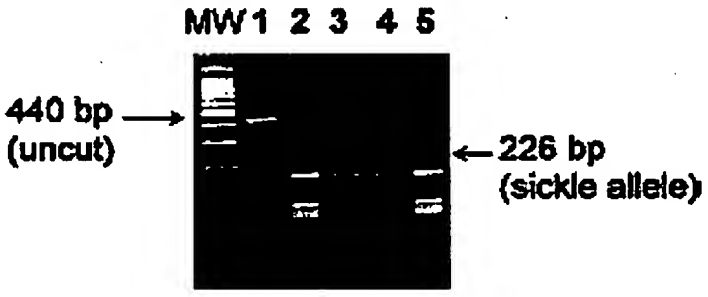

Figure 2: Expression of ß^S-globin after SFHR-mediated modification.
RNA was isolated from 4 separate experiments. RFLP analysis of RT-PCR amplicons using primer pair SC5/SC14 shows that experiment E697-1 (lane 4) has the RFLP pattern for both ß^A- and ß^S-globin sequence whereas the other experiments only show a RFLP pattern for ß^A-globin. This results confirms that SFHR-mediated modification of the genomic ß^A-globin allele results in expression of ß^S-globin

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